Intelligent Systems Based on Ordered Arrays of Biological Molecules Using the LB Technique

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ABSTRACT: The Langmuir-Blodgett (LB) technique has been used to incorporate photodynamic proteins into molecular assemblies. One technique of incorporation involves a generalized cassette attachment methodology which employs a biotin-streptavidin complexation with an electroactive polymer matrix. We have focused the efforts described here on phycobiliproteins and bacteriorhodopsin, although antibodies, enzymes, gene probes and other moieties could also be coupled into the system to build in selectivity. Photoconductivity and photobleaching of these protein systems were investigated. These results suggest that coupling these proteins, either in mixed monolayers or in multilayers, with appropriate conductive polymers or other materials will provide the opto-electronic signal transduction needed for biosensor, optical display and other applications. Initial studies involving the integration of conductive polymers into the molecular assemblies to enhance optical signal transduction are also discussed.

INTRODUCTION

BIOLOGICAL systems have evolved highly sensitive and selective optical sensors that function in vision and energy conversion to drive optical-chemical transduction processes, essential for survival. Specific examples of such systems include phycobiliprotein assemblies in algal systems and bacteriorhodopsin in bacterial cells. The phycobiliproteins, which are synthesized and assembled into complex "antennae" arrays, collect light filtering through marine or fresh waters and funnel this light in a unidirectional energy pathway into the photosynthetic reaction center in the cell to drive carbon fixation processes (Gantt, 1981; Glazer, 1985). These assemblies generally consist of at least three primary complexes: phycoerythrins, phycocyanins, and allophycocyanins. Each of these complexes has its respective absorption and emission spectra, and acts in series with an overall light energy conversion efficiency of >90%. Phycobiliproteins are used today in diagnostic reagents because of their strong fluorescence and large Stoke's shift (81 nm, 495 nm excitation and 576 nm emission for phycoerythrin) (Glazer, 1985).

Bacteriorhodopsin is a membrane protein that functions to collect light energy via a retinal moiety bound in the protein, which causes a conformational change and subsequently drives a proton gradient across the membrane. This gradient provides the chemical potential to drive the synthesis of ATP for cellular energy. Bacteriorhodopsin has been described in potential applications for optical displays, holograms, optical switching systems and other applications (Birge, 1990a, 1990b, 1993).

Our initial interest in these photodynamic protein systems was to determine if they could function as biosensors by organizing and immobilizing them into defined arrays using thin film molecular assembly techniques. The goal was to fully utilize the functional properties of these materials, with particular emphasis on optical reception, and efficiently couple them to other components, such as electroactive polymers, to enhance signal transduction. Recent studies in this area have included embedding sensing elements [e.g., enzymes (Aizawa, 1989) and DNA (Pande et al., 1993)] in conductive polymers to optimize signal transduction and maintain sensitivity and low detection limits. Our approach has involved direct linkage between the sensing element and the conductive polymer such that one may control the surface organization, density, orientation, size and spatial distribution of the elements in the assembly to optimize biosensing.

Associate Editor: D. De Rossi.

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1045-389X/94/03 0305-06 $6.00/0

Aside from the initial goal, the research has identified many potential applications for the work, including pseudo-two dimensional ordering of virtually any protein for structure studies; biosensor applications using antibodies, enzymes or gene probes; optical displays; and color mimicking. We are describing these systems as "intelligent" because they will respond to changing environmental input by effecting a change in display, color or electrical output. Devices for electronic, biomedical, protein research and optical applications are potential opportunities for the assemblies described. In addition, the ability of the methodology to selectively incorporate virtually any protein "cassette" into the assembly means that the system is essentially generic and tailorable for the specific application desired.

MATERIALS AND METHODS

Chemicals

R-phycoerythrin (PE), R-phycoerythrin biotin-XX conjugate (B-PE), and streptavidin (ST) were purchased from Molecular Probes (Eugene, Oregon). The streptavidin was received as a lyophilized powder which was reconstituted to a stock solution in Millipore MilliQ water. The biotinylated phycoerythrin was shipped in 0.1 M sodium phosphate containing 0.1 M NaCl stock solution and was used as received. The XX represents a 14 atom "spacer" attached to enhance the ability of biotin derivatives to bind to the relatively deep binding sites of streptavidin. Biotinylation was carried out by covalently binding biotin to random amide sites on the protein, resulting in three to four biotin functionalities per molecule.

Biotinylated poly(3-methanolthiophene-*co*-3-undecylthiophene (B-PMUT) was synthesized through dehydrogenation of 3-hexylthiophene and 3-methanolthiophene to form the copolymer and room temperature esterification using *N,N,*-dicyclohexylcarbodiimide and 4-pyrrolidinopyridine as catalyst for the biotinylation (Kamath et al., 1993). Bacteriorhodopsin was produced and purified from cultures of the bacterium *Halobacterium halobium* by established protocols (Oesterhelt and Stoeckenius, 1974). Additional samples of phycoerythrin and phycocyanin were prepared and purified from *Porphyridium cruentum* and *Synechococcus leopoliensis,* respectively, using established protocols (Gantt, 1981; Glazer, 1984).

Monolayers/Assemblies

Monolayer studies with the phycobiliproteins were carried out in a Lauda MGW Filmwaage trough with a surface area of approximately 930 cm^2. Pressure-area isotherms were used to determine monolayer formation and protein attachment. The B-PMUT was prepared in a 0.5 mM chloroform solution and spread over an aqueous buffered subphase containing 0.1 mM sodium phosphate and 0.1 M NaCl at pH 6.8. All subsequent experiments were carried out in this same subphase buffer. In different experiments, ST or B-PE was injected into the subphase to a final concentration of approximately 1×10^{-9} M under the B-PMUT expanded monolayer and allowed to react for two hours. In experiments for cassette designs, the reaction with streptavidin was followed by flushing with clean subphase, injection of 0.1 mg of B-PE and subsequent incubation for at least two hours. To generate pressure-area isotherms, the barrier compression rate was set at approximately 2 mm^2/min. Monolayers were transferred onto glass solid supports for characterization using the vertical dipping technique. The monolayers were stable and held at a constant pressure of 15 mN/m during transfer and resulted in approximately 100% transfer on the upstroke.

Characterization

For the phycobiliprotein monolayer assemblies, fluorescence of transferred films was measured with an argon ion laser with 10 mW laser power as the pump light source. Excitation was at 496 nm and the emission was scanned from 500 to 700 nm. Bacteriorhodopsin bulk-cast films were characterized by optical spectroscopy on a Perkin-Elmer Model Lambda 9 Spectrophotometer.

The photoconductivity measurements reported in this work were carried out on protein samples prepared by air drying a drop of an aqueous solution (for phycoerythrin and phycocyanin) or suspension (bacteriorhodopsin as purple membrane) of protein onto interdigitated electrodes (15 μm electrode fingers, 15 μm apart). These were essentially bulk-cast films which were measured to demonstrate the intrinsic photoconductivity properties of these protein systems. Photoconductivity was measured at different argon ion laser wavelengths and He-Ne laser wavelengths. The effective sampled area for photocurrent measurements was in the range of 2–3 mm in diameter. The photoconductivity was measured using a mechanically chopped laser light source in conjunction with a lock-in amplifier referenced to the chopping frequency (Figure 1). This arrangement pro-

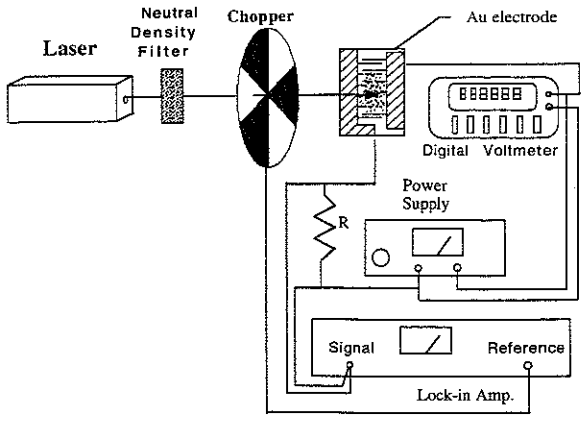

Figure 1. *Experimental setup used to collect photoconductivity measurements.*

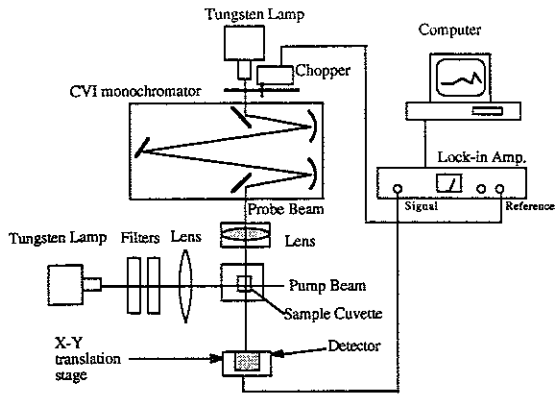

Figure 2. *Experimental setup used to conduct photobleaching measurements.*

vided a very high level of rejection of all dc leakage currents through the film, due to defects or otherwise. This was verified during the course of the experiments by blocking the laser beam and observing only small background signal levels (typically <1 pA). Thus, essentially only photocurrents were detected during these measurements. The same experimental set-up was used to evaluate both the phycobiliprotein and bacteriorhodopsin protein systems. Photoconductivity was also characterized with high intensity pulsed white light using a xenon flash lamp source. Photobleaching studies with bacteriorhodopsin were carried out by detecting the absorption signal with a photodiode detector using a phase sensitive detection method. The probe beam was chopped and the lock-in amplifier provided rejection of interference from scattered radiation from the pump beam. No blocking filters were required (Figure 2).

RESULTS AND DISCUSSION

This paper will separately address the two major components that comprise our proposed intelligent system, which is designed to optimize signal transduction. The first part will discuss a unique cassette attachment methodology used to assemble photodynamic protein and electroactive polymer into organized thin films. The second part will focus on the first demonstration of intrinsic photoelectronic response of our proteins of choice. Ultimately, the goal of this work is to integrate these components such that novel electronic and optical properties will result for a host of biosensing applications.

Cassette Attachment Methodology

The basic approach described here to establish direct linkage between the protein (sensing element) and electroactive polymer is based on biotin-streptavidin recognition. This interaction has been extensively used in biochemical assays, and research on monolayer assemblies based on this interaction has also been reported (Blankenburg et al., 1989; Darst et al., 1991; Ebersole et al., 1990; Weber et al., 1989). We have extended this approach to the cassette system through the incorporation of photodynamic proteins into an electroactive polymer matrix using a Langmuir trough. This cassette approach has been primarily used with the phycobiliprotein system and the results are presented here. However, this paper also discusses preliminary work with simple bacteriorhodopsin bulk-cast films. Ongoing experiments involve biotinylating bacteriorhodopsin and various mutants such that this cassette approach may be extended to this system as well.

Our previous studies have documented the preferential interaction of streptavidin conjugated phycoerythrin (ST-PE) with a commercially available biotinylated phospholipid (Samuelson et al., 1991, 1992a). These studies were then extended by utilizing a cassette attachment methodology where ST only was injected and incubated followed by injection of B-PE onto the biotinylated phospholipid (Samuelson et al., 1992b; Marx et al., 1993). In these studies, protein interaction was determined through increases in surface pressure upon injection/incubation of protein and the observation of a strong fluorescence signal, on transferred monolayer films, diagnostic of native phycoerythrin protein. In all cases, control runs without the appropriate combinations of reactants did not show the expansion in the isotherms or the fluorescence emission of the PE. In this study, we have substituted the biotinylated phospholipid with a novel biotinylated electroactive copolymer system. It is anticipated that this copolymer will provide improved mechanical, thermal and chemical stability as well as enhanced optical signal transduction.

Figure 3 gives an idealized schematic of the different stages in the cassette methodology which attaches B-PE onto the B-PMUT copolymer. The first stage shows an inverted LB monolayer of just the B-PMUT revealing randomly exposed biotin groups. Stage 2 shows the complexation of streptavidin to these exposed biotin groups, which still leaves accessible biotin binding sites for subsequent complexation. Stage 3 illustrates the subsequent complexation of the randomly biotinylated phycoerythrin with the free biotin binding sites of the ST, resulting in a range of possible protein orientations to the assembly. It should be noted that such an attachment methodology may be applied to any system which can be biotin derivatized, making it a very versatile technique for the incorporation of various biomolecules into an electroactive polymer matrix.

Figure 4 gives the structure of the B-PMUT copolymer and representative pressure-area isotherms for the three stages of protein attachment illustrated in Figure 3. The first curve is that of copolymer alone, the second is after injection/incubation with ST, and the third is after subsequent injection/incubation with B-PE. A comparison of these pressure-area isotherms shows an increase in area throughout the entire compression cycle as each protein is introduced to the B-PMUT monolayer. This continuous expansion suggests that the streptavidin and B-PE are each

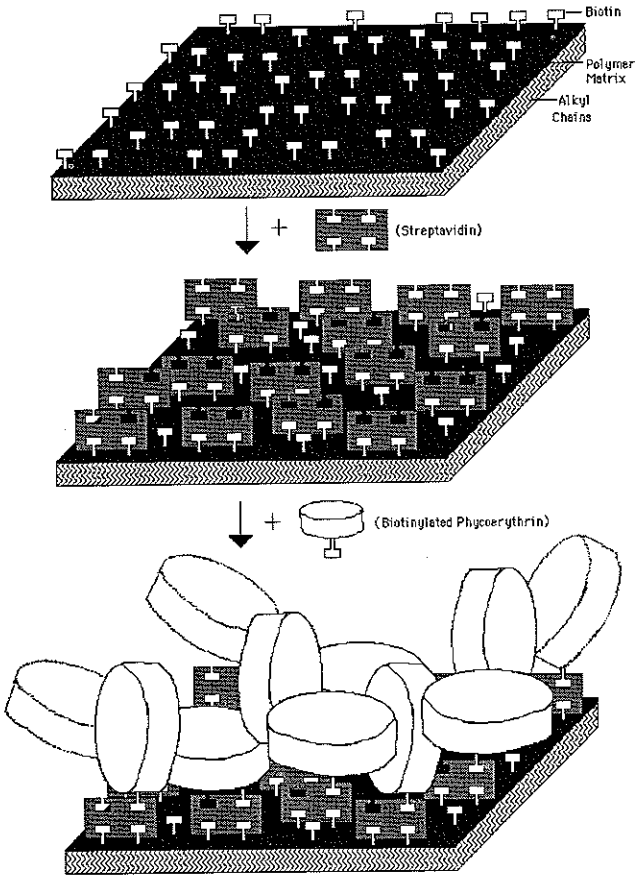

Figure 3. *Idealized schematic of the different stages that take place during the cassette attachment methodology onto the B-PMUT monolayer.*

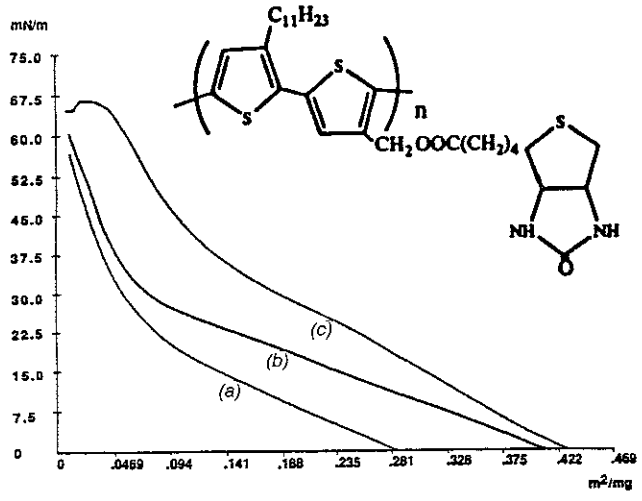

Figure 4. *Structure of the B-PMUT copolymer and pressure-area isotherms for the three stages of protein attachment: (a) B-PMUT, (b) B-PMUT/streptavidin and (c) B-PMUT/streptavidin/B-PE.*

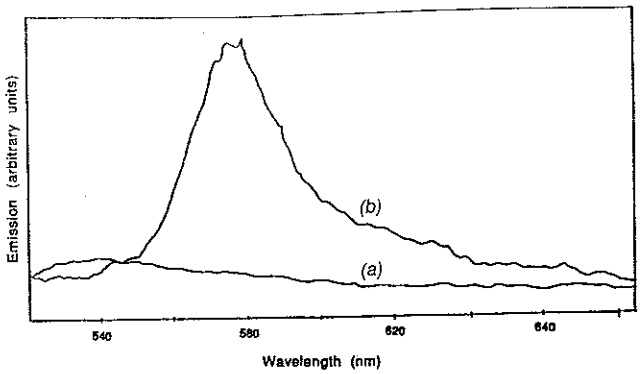

Figure 5. *Fluorescence spectra of transferred films: (a) B-PMUT/ streptavidin and (b) B-PMUT/streptavidin/B-PE.*

interacting with the monolayer film. Such behavior is not observed for control assemblies, which are each missing one component of the biotin-streptavidin complex. This suggests that specific biotin-streptavidin binding recognition of the protein systems is occurring.

To confirm the binding of the phycoerythrin to the B-PMUT monolayer, fluorescence spectroscopy was carried out on transferred LB films. Figure 5 shows the fluorescence spectrum of a monolayer of B-PMUT with only ST injected compared to a monolayer of B-PMUT with ST injected followed by B-PE. As shown, the B-PMUT/ST/B-PE monolayer gives a distinct emission peak at 576 nm. This is direct evidence of complexation of the biotinylated phycoerythrin to the monolayer and supports the cassette attachment methodology onto an electroactive polymer monolayer.

Photoconductivity

Prior studies have demonstrated photovoltaic properties of oriented thick films and Langmuir-Blodgett multilayers of bacteriorhodopsin membrane fragments (Hwang et al., 1978). Due to the charge separation during the photocycle, photoconductive properties were expected and observed for our cast films (Figure 6). When an external electrical poten-

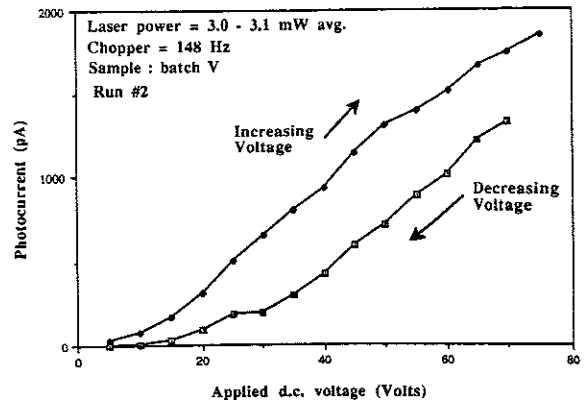

Figure 6. *Photoconductivity measurement made on bulk-cast films of bacteriorhodopsin (at 514 nm).*

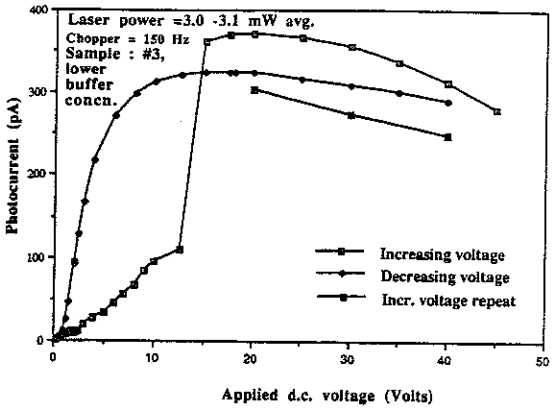

Figure 7. *Photoconductivity measurement made on bulk-cast films of phycoerythrin (at 488 nm).*

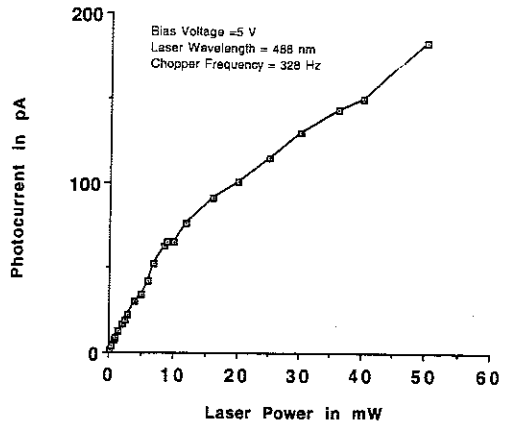

Figure 9. *Photoconductivity in phycocyanin as a function of light intensity.*

tial was applied to the film and then exposed to light, an increase in current flow was observed. In some cases, a hysteresis effect was observed, presumably due to dehydration or degradation of the sample. A point of interest is that the photocurrent increased with increased bias voltage and no saturation was observed under the experimental conditions studied.

Figure 7 illustrates the first demonstrated photoconductive response for phycoerythrin and indicates significant photocurrent. Unlike the bacteriorhodopsin response, the photocurrent in the phycoerythrin films saturates at high bias voltages. The photocurrent was observed to increase with light intensity in all protein systems to fairly high intensity levels. In phycoerythrin, the photoconductivity actually increased temporarily during this phase (Figure 8), presumably due to charge carriers generated during photodestruction and associated thermal degradation. The increase in photoconductivity with an increase in light intensity was also observed for phycocyanin and is shown in Figure 9.

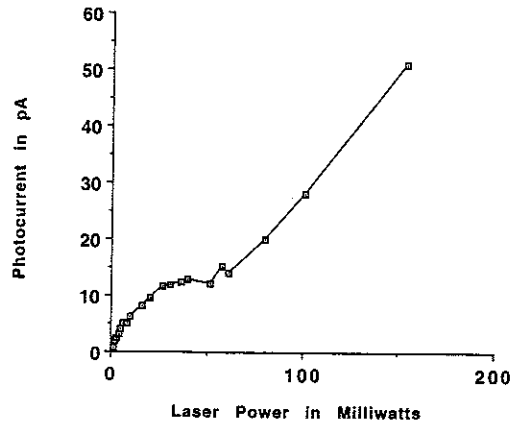

Figure 8. *Photoconductivity in a phycoerythrin bulk-cast film as a function of light intensity at room temperature, at a constant voltage bias (V_{bias} = 10 V). There are indications that sample degradation begins above 50 mW, although the photoresponse increases, at least temporarily.*

Photoconductivity measured with high intensity pulsed white light, with consideration of the limitations of the electronics used, showed response times similar to previous studies with high resistance LB films, and slower than that with low resistance LB films. Submillisecond pulses were detected despite the overall response times (on the order of hundreds of milliseconds) under these limiting conditions. Reversible photobleaching of bacteriorhodopsin films can be enhanced under appropriate conditions for potential optical display applications. Figure 9 illustrates the photobleaching effect observed in our studies. Similar effects have been observed in thick films incorporating the same reagents in an agarose gel medium.

We have thus observed, for the first time, photoconductivity in cast films of the phycobiliproteins, phycoerythrin

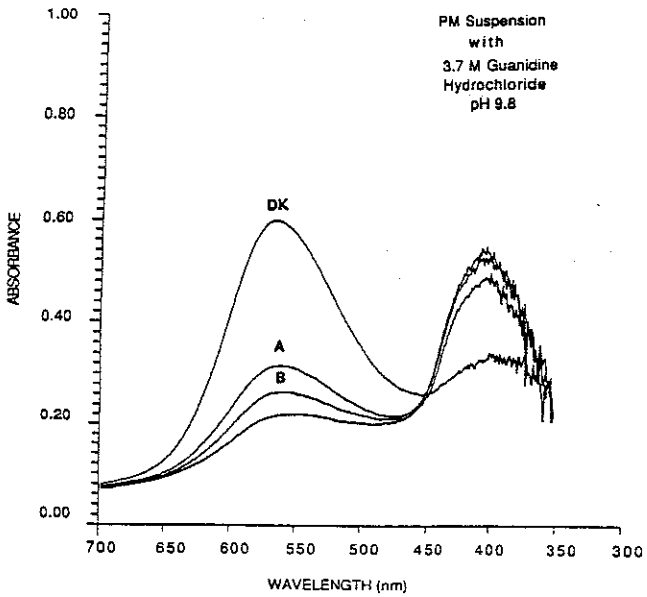

Figure 10. *Example of the photobleaching effect observed with suspensions of bacteriorhodopsin (as membrane fragments) prepared with 3.7 M guanidine hydrochloride at pH 9.8. The light intensity increases as DK(dark) < A < B < E.*

and phycocyanin. This effect is presumably associated with the tetrapyrrole chromophore moiety, although the detailed mechanism has not yet been elucidated. The temperature dependence of the photoconductivity in phycoerythrin (Beladakere et al., 1993) suggests that the photogenerated charge carriers are transported through the film by a hopping conduction mechanism. These protein systems, in addition to the well-known photodynamic systems such as bacteriorhodopsin or chlorophyll, thus offer opportunities in photoelectronic signal transduction. Therefore, direct "ordered" linkage of these systems to an intrinsically conductive matrix (using our cassette attachment methodology) should form the basis for a variety of novel intelligent materials and devices.

ACKNOWLEDGEMENT

Support from ARO Grant DAAL03-91-G-0064 is gratefully acknowledged. We also thank R. Sarma for LB assistance. S. Sengupta and M. Cazeca would also like to acknowledge support from SBIR Grant DAAK60-91-C-0035.

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